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Molecular Mechanisms of Prostate Cancer Progression

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**13. ABSTRACT (Maximum 200 Words)**

In studies to define the mechanisms involved in the progression of immortal, non-tumorigenic prostate cells to a tumorigenic state, we have found that molecular chaperones are elevated along with telomerase activity. Elevated chaperone function results in an increase in telomerase assembly and is associated with prostate cancer progression. In order to determine the importance of the chaperone increase, we are investigating, both genetically and pharmacologically, whether ectopic chaperone expression results in transformation and whether chaperones are targets for prostate cancer therapy. The hsf-1 transcription factor has been over-expressed in non-tumorigenic prostate cells, resulting in increased hsp90 and hsp70 expression and an upregulation of telomerase.Preliminary data suggests that exogenous hsf-1 has little effect on tumorigenic growth. Using both a pharmacologic (radicicol, a specific hsp90 inhibitor) and genetic (siRNA to hsp90) approaches, malignant prostate cancer cell lines show only a transient decline in telomerase activity but a significant decrease in telomere length, suggesting that chaperones function at the telomere independent of their interaction with telomerase.

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Telomerase, chaperone, tumors, transformation, hsp90, radicicol

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CONTRACTING ORGANIZATION: Virginia Commonwealth University
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## Introduction

Telomerase is a cellular reverse transcriptase that is associated with over 90% of human prostate cancers and is composed of 2 integral components, an RNA template (hTR - human Telomerase RNA) and a catalytic polymerase (hTERT - human TElomerase Reverse Transcriptase) (Weinrich et al 1997). We have discovered that telomerase assembly is modulated by its interaction with the molecular chaperones in the hsp90 family, including hsp90, hsp70, p23 and hsp40 (Holt, et al., 1999) (a model of telomerase assembly is shown in Figure 1). Using a highly relevant model for prostate cancer progression with non-tumorigenic, tumorigenic, and metastatic sublines with the same genetic background, we found that tumorigenic cells had 10-15-fold higher levels of telomerase activity than did the non-tumorigenic line and that there was no change in the expression profiles of the telomerase components, hTR and hTERT (Akalin, et al., 2001). We also found that the molecular chaperone family of proteins were upregulated as cell progressed to a more tumorigenic state, suggesting that the upregulation of telomerase activity is due to elevated assembly of the enzyme, rather than an increase of individual telomerase components. In fact, when purified chaperones are incubated with an extract from the non-tumorigenic line, telomerase is elevated, suggesting that this cell line harbors a significant amount of unfolded or improperly folded telomerase.

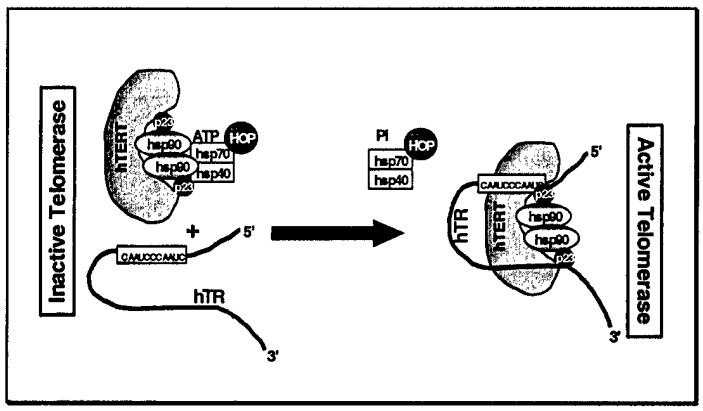


**Figure 1. The hsp90 complex is required for assembly of active telomerase.** Our working model for the chaperone-mediated ordered assembly of active human telomerase. [hTR - human telomerase RNA; hTERT - human telomerase reverse transcriptase]

## Body

Normal human prostate epithelial cells were immortalized by expression with the SV40 large T antigen oncogene, and an immortal, non-tumorigenic, telomerase-positive cell line was selected, P69. When injected into nude mice, no tumors formed within the standard 8-12 weeks, but if left in the animal for 6 months, 2 palpable sporadic tumors from a total of 19 mice formed after in vivo selection (Bae et al., 1994). Both lines, M2205 and M2182, were propagated in culture and reinjected into mice and were found to be tumorigenic. After undergoing another round of selection, the metastatic subline, M12, was generated. As our model for prostate cancer, the P69-M12 progression scheme provides an excellent system from a defined genetic background to study the molecular and cellular changes that occur during prostate cancer progression. Having found elevated telomerase levels in the more advanced prostate cancer lines and tumor samples (Akalin et al., 2001), our data indicates that this change in activity is due to an increase chaperone-mediated telomerase assembly rather than expression of the hTERT and hTR core components of the telomerase holoenzyme. As such, our goals are to determine if chaperones are the cause of the transformation event during prostate cancer progression and to show that chaperones are likely targets for anti-telomerase therapy in advanced prostate cancer.

*Objective #1*: Define the role of chaperones and telomerase activation during prostate cancer progression.

This first aim is designed to determine if the elevated chaperone expression levels can cause transformation in the non-tumorigenic P69 cells. We have designed and made retroviral constructs for chaperone-related genes and hTERT, as reported last year. Each construct can be utilized and stably selected into cells either alone or in combination with any of the other constructs.

Each construct was transiently transfected into the amphotropic mouse cell line, Phoenix A, the supernatant was harvested at 48h post-transfection, and the P69 cell line was infected with each of the constructs. The appropriate selection was accomplished and compared to uninfected controls, followed by recovery of pooled clonal populations. We reported last year that infection of hsp90 and p23 showed a modest, but reproducible increase in telomerase activity. Because the p23 population showed a modest increase that was suggestive of increased chaperone function (similar to the hsf-1 and hsp90 populations, not shown), we obtained single-cell-derived clones for each of the infected populations. We find some clonal heterogeneity with the p23 clones, ranging from 2-fold to 10-fold increased activity, suggesting that p23 is perhaps the limiting factor in the telomerase assembly process in P69 cells (as we reported last year). One of the problems that was encountered was a lack of elevated hsp90 in our clones, suggesting a feedback inhibition of endogenous hsp90 in these cells. As such, we switched our focus to the heat shock transcription factor, hsf-1. Over-expression of hsf-1 in P69 cells results in an increase in hsp90, hsp70, hsp27, and hsf-1 (Figure 2), and importantly for our studies, telomerase activity is also reproducibly increased in these cells after hsf-1 expression (Figure 2).

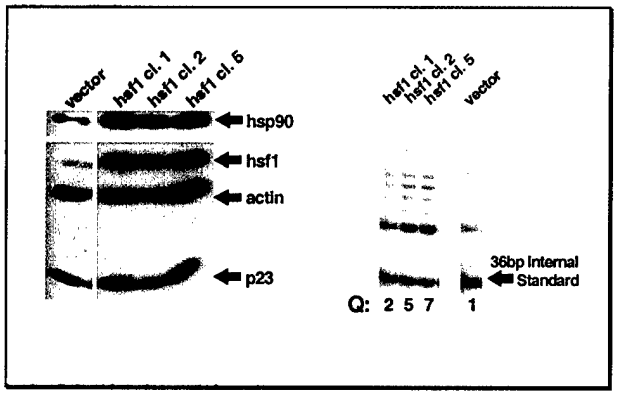


**Figure 2. Effects of hsf-1 overexpression in P69 cells.** Over-expression of the heat shock transcription factor, hsf-1, induces expression of HSP90 and p23 (as well as HSP70), and results in a corresponding increase in telomerase activity in P69 cells ('Q' - quantitation).

We continue to follow these hsf-1 clones for growth and transforming abilities. A very preliminary experiment suggests that over-expression of hsf-1 does not result in transformation of the P69 cell population. Using the hTERT over-expressing cells, we have also found no observable change in the transforming capabilities of the P69 cells, indicating that telomerase alone is not a transforming gene. However, if oncogenic ras (RasV12) is infected into P69 cells, significant colonies are found in a soft agar assay. In addition, the rasV12-P69 cells, although transformed, do not have a substantial increase in telomerase activity nor do they upregulate the hsp90, hsp70, or p23 chaperones. Because these data are very preliminary, we do not show them here, but these cells continue to be under investigation.

*Objective #2:* Determine the cellular and molecular consequences of targeted inhibition of chaperones and/or telomerase using pharmacological and genetic approaches in tumorigenic prostate cancer cells.

Because we observe an increase in chaperone expression and function in prostate cancer cell lines and primary prostate tumors (Akalin et al., 2001), our goal was to determine if chaperones, specifically hsp90, were targets for inhibition of telomerase activity and reversion of the tumorigenic phenotype to a less severe, non-tumorigenic state. Thus far, we have successfully employed both the pharmacological and genetic approaches to blocking chaperones using drugs (radicicol and geldanamycin) and siRNA to block hsp90 function. Initially, we found that geldanmycin was quite toxic and resulted in a decline in telomerase as well as in cell viability, an outcome that was undesirable for an indirect method of telomerase inhibition (data not shown). As we reported last year, radicicol, which binds in the ATP-binding pocket of hsp90 in a similar manner as geldanamycin and blocks hsp90s ability to associate with p23 and other chaperone targets, was capable of blocking telomerase activity in a time-dependent manner. Using a chronic continuous replenishment of radicicol in the M12 cells, we found that telomerase activity (hence, chaperone function) was reduced but only in a transient fashion (Figure 3). We also observe a decrease in hsp90 function in the form of a decline in p23 levels (p23 stability is reduced due to its inability to interact with hsp90) (Figure 3).

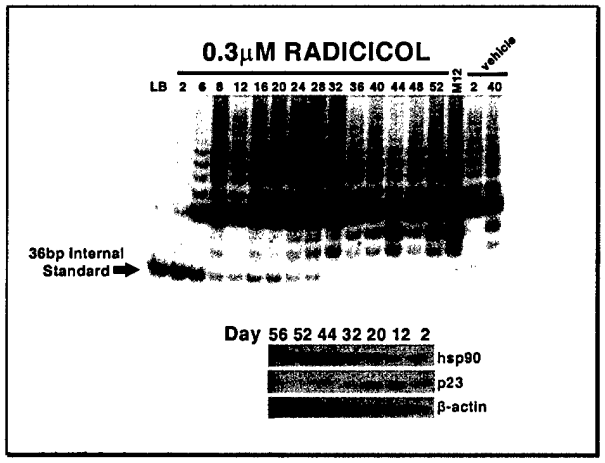


**Figure 3. Transient inhibition of telomerase activity in the metastatic M12 prostate cancer subline after treatment with the hsp90 inhibitor, radicicol.** Chronic treatment of M12 cells with radicicol (0.3μM) results in a transient inhibition of telomerase activity without observable cell death or changes in growth rate. The bottom panel (Western) shows a decrease in p23 levels over time, suggesting a lack of hsp90 function.

We have targeted hsp90 genetically as well, initially with antisense, which was unsuccessful, and more recently with siRNA for hsp90. Figure 4 shows that although there is no decline in telomerase activity over time in the hsp90 siRNA cell clones, hsp90 is nearly undetectable in these cells (note the Western blot below the telomerase activity (TRAP) autorad). This is consistent with the transient down-regulation of telomerase in the radicicol treated cells in that there was likely an initial decline in telomerase activity after infection of the siRNA for hsp90, but this decrease was not observable as it would have occurred during selection of stable populations of cells expressing the hsp90 siRNA. Thus, we conclude that there is only a transient down-regulation of telomerase activity in prostate tumor cells treated with hsp90 inhibitors.

Because of these promising initial results, cells were treated on differing regimens with radicicol for upwards of 3 months. Our initial pass with either 2-day or 4-day replenishment of drug

provides strikingly novel results with an expected long-term outcome. As discussed in last year's report, the 2-day treatments lead to a reduction in functional telomerase at the beginning of treatment, followed by a recovery of activity, which mimics the observed decline in chaperone levels, while the 4-day treatments again show the transient telomerase decline in the absence of chaperone reduction or telomere shortening.

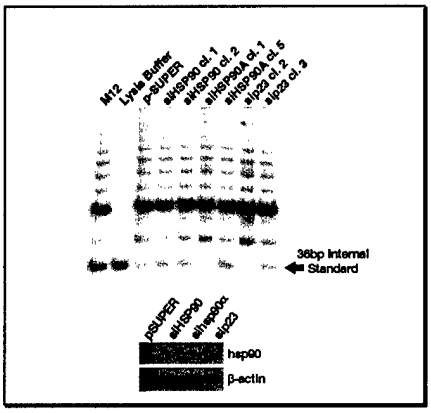


**Figure 4. Metastatic prostate cancer cells with genetic inhibition of HSP90 show no decline in telomerase activity.** M12 cells were infected with stable siRNA directed against hsp90α/β, hsp90α, or p23. Protein levels of chaperones (only hsp90 is shown) are reduced after infection (Western blot in the lower panel) and telomerase activity was observed in all clones (TRAP assay shown in the upper panel).

Even though telomerase is not altered in hsp90 inhibited cells, telomeres still shorten over time in both the pharmacologic and genetic hsp90 inhibition studies (Figure 5). This gradual telomere shortening eventually results in about a 1.5kbp loss of telomeric DNA, which is consistent with either a reprogramming of senescence or induction of apoptosis, a more likely event given the presence of T antigen in these cells (inactivation of p53 and pRB, both of which are required for senescence). Interestingly, the hsp90 siRNA cells show no signs of a decline in growth, even over approximately 12 weeks in culture. While interesting, we will determine if inhibition of hsp90 in prostate cancer cells will sensitize the cells to other therapeutic compounds.

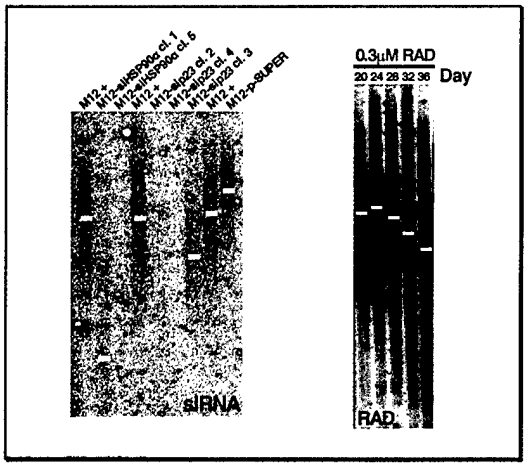


**Figure 5. Telomere shortening is observed in prostate cancer cells genetically or pharmacologically targeting HSP90.** M12 cells were stably infected with siRNA or continuously cultured in media containing 0.3mM radicicol. DNA was isolated and telomere length assessed by Southern with a telomere specific probe.

Long-term treatments with radicicol (out to 55 days) using the 2-day treatments provided a surprising, but somewhat expected result: sudden apoptosis at day 55. This apoptotic timing is completely reproducible (at least 5 independent experiments) and virtually 100% of the cells undergo apoptosis within the 53-55 day window. We are further characterizing the timing of this apoptotic effect and the molecular and cellular consequences of chaperone inhibition with respect to telomerase activity and telomere erosion. One mechanism that we are exploring is how

telomeres can continue to shorten in the presence of active telomerase. Many possibilities exist, including telomere accessibility, subcellular localization, and post-translation modification. In addition, we have begun to utilize new compounds (e.g. novobiocin) to block chaperone function using a totally different mechanism of hsp90 inhibition to determine if the cellular and molecular consequences observed for the pharmacologic inhibitors of molecular chaperones are specific for chaperones or telomerase or require an interaction of both. One of the possible explanations for our observed telomere shortening in the absence of telomerase inhibition is that chaperones function at the telomere independent of telomerase. It is plausible that hsp90 is capable of interacting with telomere binding proteins, which include TRF1, TRF2, and a host of DNA repair complexes, and that this interaction is disrupted, resulting in destabilization of telomere structure and function. In fact, we have found that hsp90 and p23 interact with the telomere (Figure 6) using a chromatin immunoprecipitation assay, which allows one to show protein/DNA interactions in extracts isolated from actively dividing (or treated) cells. We are currently exploring the possibility that hsp90 plays a role in a telomerase-independent telomere function.

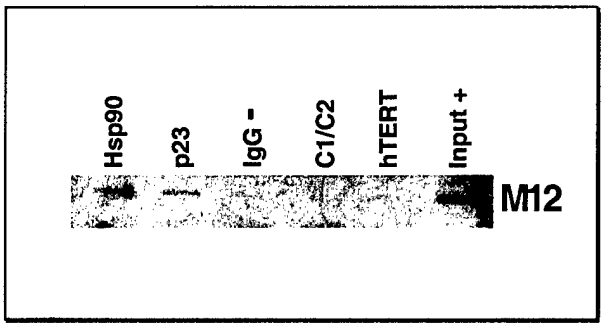


**Figure 6. Chaperones HSP90 and p23 are found at telomeres.** Telomeric sequences are detected using a telomere probe following chromatin immunoprecipitation with antibodies for HSP90 and p23 (IgG and hnRNP C1/C2 antibodies served as negative controls).

**Key Research Accomplishments**

1-establishment of chaperone-expressing P69 cells that result in an increase in telomerase activity.

2-expression of molecular chaperones in non-tumorigenic cells results in a partial feedback inhibition of chaperone expression (will be further defined in terms of exogenous versus endogenous protein expression).

3-blocking chaperone function with radicicol results in transient telomerase inhibition, telomere erosion, and eventually cell death in tumorigenic cell lines, indicating that targeting chaperones in tumorigenic prostate cancer cells may be an appropriate therapy.

**Recommended Changes to the Proposed Work Based on Additional Findings**

We have over-expressed hsf-1 in the P69, non-tumorigenic cells and have shown an increase in telomerase activity. Additional experiments may include expressing hsf-1 phosphorylation mutants or blocking certain hsf-1 kinases in P69 cells, which would allow us to eliminate the feedback inhibition of hsp90 over-expression by having constituitively active hsf-1 and may result in the activation of other hsps and telomerase. We will also expand our work to include the investigation of this mechanism for chaperone suppression, as well as expand it to include ras-induced transformation of the P69 cells to determine if chaperone and telomerase expression profiles are similar to the in vivo selected sublines. In addition, we have expanded this work to

include the hTERT overexpression and the mechanisms involved in how elevated hTERT would provide and increase in telomerase activity. In the second aim, we originally proposed utilizing antisense gene expression to genetically block chaperone expression in the tumorigenic lines. However, since the application was submitted, we have utilized small interfering RNAs to block to functions of telomerase and p53 in other cells lines and strains, and we have shown that we can successfully employ this technology to our hsp90 system. In addition within this aim, we will determine the nature of the hsp90/telomere association and the telomere proteins involved in the telomerase-independent telomere shortening that occurs in the metastic subline, M12.

## Reportable Outcomes

*Manuscripts*

None, although 4 are in preparation

*Abstracts/Presentations*

Harvey,S.A., L.W.Elmore, and **S.E.Holt**. AACR: The role of telomeres and telomerase in cancer, San Francisco, CA. December 2002.

**Holt,S.E.,** S.A.Compton, K.O.Jensen, and L.W.Elmore. CaP CURE. New York, NY. November 2003.

*Invited Seminars*

**Holt,S.E.** Department of Biological Sciences, Texas Tech University, Lubbock, TX. October 2003.
**Holt,S.E.** Department of Human Genetics, MCV/VCU, Richmond, VA. September 2003.
**Holt,S.E.** Mini-Medical School, Science Museum of Virginia, Richmond, VA. March 2003.
**Holt,S.E.** Department of Neurooncology, MCV/VCU, Richmond, VA. March 2003.
**Holt,S.E.** Department of Biology, Maggie Walker Governor's School, Richmond, VA. March 2003.

*Development of Cell Lines*

We have developed cell lines for telomerase over-expression in the non-tumorigenic prostate epithelial cell line, as well as individual P69 cell lines with oncogenic ras, hsp90, hsf-1, and p23 over-expression. We have also made the corresponding tumorigenic cell lines knocking out hsp90, p23, telomerase, and eventually hsf-1 using the siRNA approach.

*Funding Applied For*

Department of Defense Breast Cancer Research Program, IDEA award, May 2003 – Awarded
Department of Defense Prostate Cancer Research Program, Hypothesis award, March 2003 – Declined
National Institutes of Health (NCI), June 2003 – Declined
American Cancer Society, October 2003 – in review
Department of Defense Breast Cancer Research Program, Predoctoral award, May 2003 – Awarded

## Conclusions

Having established numerous cell lines with over-expression of chaperone-related genes, telomerase, and oncogenic ras in the non-tumorigenic P69 cell line, as well as siRNA inhibition of hsp90, p23, and telomerase, we are clearly on pace to define the cellular consequences of ectopic expression or inhibition of these proteins and their role in transformation. Our data conclusively shows that over-expression of telomerase on its own is not sufficient to elicit transformation nor does it allow for elevation of the chaperone proteins. Increased chaperone levels after hsf-1 expression has provided promising results related to stable expression and transformation. Our results for the pharmacologic and genetic inhibition of molecular chaperones and telomerase is not only interesting, but represents the first indirect method for a telomerase-

independent, chaperone-mediated telomere shortening in a prostate cancer cell model system. Inhibition of chaperone function in tumorigenic prostate cells may represent a novel mode of prostate cancer therapy that would be useful for patients with more severe disease, which may provide a means of limiting recurrence or metastasis.

**References (bold** indicates PI authored papers)


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**Harvey,S.A., K.O.Jensen, L.W.Elmore, and S.E.Holt. 2002. Pharmacological approaches to defining the role of chaperones in aging and prostate cancer progression. Cell Stress and Chaperones 7:230-234.**

**Holt,S.E., D.L.Aisner, J.Baur, V.M.Tesmer, M.Dy, M.Ouellette, D.O.Toft, J.B.Trager, G.B.Morin, W.E.Wright, J.W.Shay, and M.A.White. 1999. Functional requirement of p23 and hsp90 in telomerase complexes. Genes and Development 13:817-826**

**Weinrich,S.L., R.Pruzan, L.Ma, M.Ouellette, V.M.Tesmer, S.E.Holt, A.G.Bodnar, S.Lichtsteiner, N.W.Kim, J.B.Trager, R.D.Taylor, R.Carlos, W.H.Andrews, W.E.Wright, J.W.Shay, C.B.Harley, and G.B.Morin. 1997. Reconstitution of human telomerase with the template RNA hTR and the catalytic protein subunit hTRT. Nature Genetics 17: 498-502.**

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# Appendix Cover Sheet

## BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed on Form Page 2.
Photocopy this page or follow this format for each person.

| NAME | POSITION TITLE |
|---|---|
| Shawn E. Holt, Ph.D. | Associate Professor |

EDUCATION/TRAINING (*Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.*)

| INSTITUTION AND LOCATION | DEGREE (*if applicable*) | YEAR(s) | FIELD OF STUDY |
|---|---|---|---|
| The Colorado College, Colorado Springs, CO | B.A. | 1985-1989 | Biology |
| Texas A&M University, College Station, TX | Ph.D. | 1989-1994 | Genetics |
| The University of Texas Southwestern, Dallas, TX | Postdoc | 1994-1998 | Aging and Cancer |

RESEARCH AND PROFESSIONAL EXPERIENCE:, INCLUDING GRANT SUPPORT. DO NOT EXCEED 3 PAGES.

| | |
|---|---|
| 1998-2003 | Assistant Professor, Department of Pathology and Department of Human Genetics, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA |
| 2003-present | Associate Professor (collateral track), Department of Pathology and Department of Human Genetics, MCV/VCU, Richmond, VA |
| 1998-present | Member, Massey Cancer Center, Virginia Commonwealth University/Medical College of Virginia, Richmond, VA |
| 2002-present | Adjunct Faculty, Department of Pharmacology and Toxicology, MCV/VCU, Richmond, VA |
| 2002-present | Member, Molecular Biology and Genetics Program, MCV/VCU, Richmond, VA |
| 2003-present | Director, Graduate Studies and Education, MCV/VCU, Richmond, VA |

**Awards and Honors:**

| | |
|---|---|
| 2000-2003 | The V Foundation Scholars Program, Cary, NC ($100,000 award) |
| 1996-1998 | NRSA Fellowship, National Institute on Aging, while at UT Southwestern, Dallas, TX |
| 1994 | Outstanding Presenter, Research Symposium, Texas A&M University, College Station, TX |
| 1994 | Outstanding Student Government Member, Texas A&M University, College Station, TX |
| 1988-1989 | Dean's List, The Colorado College, Colorado Springs, CO |
| 1988 | Most Dedicated Football Player, The Colorado College, Colorado Springs, CO |
| 1987 | Rookie of the Year, Baseball, The Colorado College, Colorado Springs, CO |
| 1985-1987 | Outstanding College Students of America |

**Publications (over the past 3 years, from a total of 38)**

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**1**-Busbee,D., S.Miller, M.Schroeder, V.Srivastava, B.Guntapalli, E.Merriam, **S.Holt**, V.Wilson, and R.Hart. 1995. DNA polymerase $\alpha$ Function and Fidelity: Dietary restriction as it affects age-related enzyme changes. International Life Sciences Institute; Risk Assesment Symposium.

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**Existing/Pending Support**

**Active**

P.I.: David A. Gewirtz, Ph.D. (Shawn E. Holt, Ph.D., Co-PI, 10% effort)
Title: Reciprocal regulation of senescence/apoptosis in response to adriamycin in the breast tumor cell
Agency: Department of Defense

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Prostate Cancer Transformation
Agency: Department of Defense

Agency: NIEHS (ES03828)
Investigator: Shawn E. Holt, Ph.D. (pilot project funding)

P.I.: Colleen Jackson-Cook, Ph.D. (Shawn E. Holt, Ph.D., Co-PI, 10% effort)
Title: Aging and genomic changes: role of enviroment/genetics
Agency: NIH

P.I.: Shawn E. Holt, Ph.D.
Title: Defining the regulation of telomerase through identification of mammary-specific telomerase interacting proteins
Agency: Department of Defense Breast Cancer program

**Pending**

P.I.: Shawn E. Holt, Ph.D.
Title: Mechanisms of Drug-Induced Telomere Dysfunction
Agency: American Cancer Society